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The protective antigen and lethal factor components of anthrax toxin were isolated from Bacillus anthracis culture supernatants and separated using dye-ligand affinity chromatography. The two components were assayed for serological activity by immunodiffusion analysis, for molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and for biological activity by injection into Fisher 344 rats. They were shown to be serologically distinct, to possess different electrophoretic mobilities, and to demonstrate lethal toxic activity only when combined.

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USE OF DYE-LIGAND AFFINITY CHROMATOGRAPHY FOR SEPARATION OF TOXIN COMPONENTS OF BACILLUS ANTHRACIS

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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Anthrax continues to occur in humans as a result of exposure to infected animals or animal products, such as hides, wool, meat, or bones. Cutaneous anthrax accounts for most human cases of the disease, and its response to treatment with antibiotics is quite favorable. Gastrointestinal and pulmonary forms of the disease, however, are usually fatal. There is currently available a licensed anthrax vaccine for human use which appears to afford some protection against the disease [1, 2]. The vaccine consists of alum-precipitated supernatant material from cultures of B. anthracis. However, an improved vaccine is desirable because the present vaccine is reactogenic, the recommended vaccination regimen is time-consuming and requires yearly boosters, and the vaccine itself consists of a crude culture supernatant preparation. Isolation of the three purified immunogenic components of the anthrax toxin, edema factor (EF), protective antigen (PA), and lethal factor (LF), is necessary for the study and development of improved vaccine preparations. Methods previously used for the isolation and purification of anthrax toxin components have included ultracentrifugation [3], adsorption on sintered-glass filters [4, 5], chromatography using ion exchange cellulose [3, 6], polyacrylamide [5], sephadex [5], or hydroxyapatite [7, 8], precipitation using trichloracetic acid [9] or ammonium sulfate [10], and isotachophoresis and high pressure liquid chromatography [11]. This paper describes the application of dye-ligand affinity chromatography to the study of B. anthracis toxin components.

Dye-ligand chromatography, a variant of affinity chromatography, utilizes synthetic textile dyes as immobilized ligands. The dye-ligands employed here consist of various triazine dyes coupled to a cross-linked 5% agarose support matrix. In preliminary studies it was determined that the most effective separation of PA and LF from crude anthrax toxin was achieved with the Green A

dye-ligand affinity column, and thus only the data pertaining to this dye will be presented. Characterization of isolated fractions was made by measuring their biological lethal activities as well as their serological and physical differences.

MATERIALS AND METHODS

Anthrax toxin was produced from the Vollum 1B strain of B. anthracis using the method of Ristroph and Ivins [12]. Toxin was dialyzed against 20 mM Tris/HCl, pH 7.5 (Trizma base, BioRad Laboratories) at 4°C for 18 h before use. Dye-ligand affinity chromatography columns, supplied as a dye matrix screening kit consisting of one 2-ml column for each of five different dye-ligands plus an agarose control column (Amicon Corp.), were regenerated at room temperature with 10 ml 8 M urea. The columns were then equilibrated with 10 ml starting buffer (20 mM Tris/HCl, pH 7.5) at 4°C. Two milliliters of crude toxin containing approximately 40 µg protein per ml were passed into each column. The buffer flow was then halted for 30 min to allow binding of components to the dye-ligand. Two more 2 ml samples of toxin were added, each followed by a 30 min binding period. The columns were washed with 10 ml of starting buffer, and bound proteins were then eluted with a second buffer (20 mM Tris/HCl, pH 7.5, 1.5 M KCl). The 6 ml of column effluent collected during application of toxin to the column were pooled with the first 2 ml of wash with starting buffer and designated drop-through/wash (DTW). The first 8 ml of eluate were pooled and designated EL.

Assay of lethal toxic activity of DTW and EL fractions was performed in Fisher 344 rats by the method of Haines, et al. [13]. The EL pools were dialyzed against starting buffer at 4°C for 18 h to reduce toxic levels of potassium. Three milliliters of whole toxin, DTW, or EL, or 4 ml of a 1:1 mixture of DTW and EL were injected into the penile vein of the rats, the times till death recorded, and the toxic units (TU) per ml determined [13].

The method of Thorne and Belton [14] was used for immunodiffusion analysis of the fractions. Anti-PA antiserum was raised in goats immunized with an antigen preparation produced by the Michigan Department of Public Health and consisting predominantly of the PA toxin component from the V770-NP1-R strain of B. anthracis. This antigen preparation was termed Michigan antigen (MA). Anti-LF antiserum was obtained by absorbing antiserum from goats immunized with B. anthracis Sterne strain spores with MA. After incubation for 2 days at room temperature the plates were rinsed with several changes of 0.15M NaCl, once in distilled water, then treated with 10% methanol (v/v) for 5 min. The plates were stained with 0.025% Coomassie blue in 10% acetic acid for 15 min. After destaining with 10% methanol the gels were photographed.

Sodium dodecyl sulfate vertical slab gel electrophoresis of fractions was performed by the method of Laemmli [15]. Gels were stained by a silver stain method which is 100-fold more sensitive than Coomassie blue [16].

RESULTS AND DISCUSSION

Table I presents results of three experiments measuring the lethal toxic activity of DTW and EL fractions in Fisher 344 rats. Because of the small capacity of the dye-ligand columns, only one rat in each fraction pool could be injected. With the Green A column no deaths occurred in either the DTW or EL fraction pools. However, the combination of DTW and EL fractions was lethal. Since the presence of both PA and LF are required for lethal activity of anthrax toxin [4, 6], it appears that the two factors were separable by chromatography on the Green A column.

Immunodiffusion analysis results of the Green A DTW and EL fraction pools are shown in Fig. 1. Anti-PA antiserum formed a precipitin line against crude

whole toxin, MA, and EL, but not against DTW. With anti-LF antiserum the LF component was found in whole toxin and DTW but not in EL.

Fig. 2 presents results from sodium dodecyl sulfate vertical slab gel electrophoresis. Lane 1 contains the high molecular weight standards. Lane 5 contains EL, which corresponds to the position of MA in Lane 3. The upper band of whole toxin in Lane 2 thus appears to be the PA component. The principal band of DTW in Lane 4 occupies a lower position than the PA component on the gel and is presumably LF. The molecular weights of the PA and LF components of anthrax toxin are 85,000 and 83,000 daltons, respectively (S. Leppla, personal communication).

In conclusion, separation of toxin components PA and LF from B. anthracis crude whole toxin is achieved by dye-ligand affinity chromatography with the Green A dye. The two isolated fractions are serologically distinct as identified by immunodiffusion analysis and possess different molecular weights as determined by SDS polyacrylamide gel electrophoresis. The electrophoretic similarity between EL and MA, results of biological activity of the fractions in rats and serological identification with specific antiserum suggests that EL contains the PA component and that DTW contains LF.

Dye-ligand affinity chromatography is a promising new technique for the separation and isolation of components of B. anthracis toxin. The synthetic dyes are resistant to chemical and enzymatic attack and the triazine-agarose media is reusable. The separation efficiency is very much dependent upon both the activity of the whole toxin as well as the lot number of the dye.

Purification of the EF component of anthrax toxin by dye-ligand affinity chromatography is currently being investigated.

LITERATURE CITED

1. P. S. Brachman, H. Gold, S. A. Plotkin, F. R. Fekety, M. Werrin, and N. R. Ingraham, Am. J. Pub. Hlth., 52 (1962) 632.
2. G. G. Wright, T. W. Green, and R. G. Kanode, Jr., J. Immunol., 73 (1954) 387.
3. K. F. Heumann and J. F. A. McManns (editors), Conference on Progress in the Understanding of Anthrax, Fed. Proc., Bethesda, Md., October 31 and November 1, 1966, Federation of American Societies for Experimental Biology, Bethesda, Md., 1967.
4. F. A. Beall, M. J. Taylor and C. B. Thorne, J. Bacteriol., 83 (1962), 1274.
5. D. C. Fish, B. G. Mahlandt, J. P. Dobbs, and R. E. Lincoln. J. Bacteriol., 95 (1968) 907.
6. J. L. Stanley and H. Smith, J. Gen. Microbiol., 26 (1961) 49.
7. S. H. Leppla, Proc. Nat'l Acad. Sci., 79 (1982) 3162.
8. H. Smith and J. L. Stanley. J. Gen. Microbiol., 29 (1962) 517.
9. R. E. Strange and F. C. Belton, Bact. J. Exptl. Pathol., 35 (1954) 153.
10. C. B. Thorne, D. M. Molnar, and R. E. Strange, J. Bacteriol., 79 (1960) 450.
11. C. Halloway (Editor), Analytical Isotachophoresis, Proceedings of the Third International Symposium on Isotachophoresis, Hannover, Germany, June 1-4, 1982, Elsevier Scientific Publishing Company, Amsterdam (In press).
12. J. D. Ristroph and B. E. Ivins, Infect. Immunol., 39 (1983) 483.
13. B. W. Haines, F. Klein and R. E. Lincoln, J. Bacteriol., 89 (1965) 74.
14. C. B. Thorne and F. C. Belton, J. Gen. Microbiol., 17 (1957) 505.

15. U. K. Laemmli, Nature, 227 (1970) 680.

16. J. H. Morrissey, Anal. Biochem., 117 (1981) 307.

TABLE I

BIOLOGICAL ACTIVITY OF FRACTIONS FROM GREEN A DYE-LIGAND COLUMNS IN FISHER 344 MALE RATS

COLUMN	FRACTION	TU/ml[a] Experiment[b] 1	2	3	AVERAGE PROTEIN µG/ml
Toxin	---	69.3	90.5	12.3	38.3 ± 15.30
Control	DTW	46.3	31.2	13.4	28.7 ± 12.68
	EL	No death			5.1 ± 5.25
	DTW + EL	11.6	14.6	5.5	ND[c]
Green A	DTW	No death			9.7 ± 5.45
	EL	No death			22.8 ± 10.97
	DTW + EL	15.3	15.3	10.7	ND

[a]Toxic units per ml were determined by the method of Haines et al. [13].

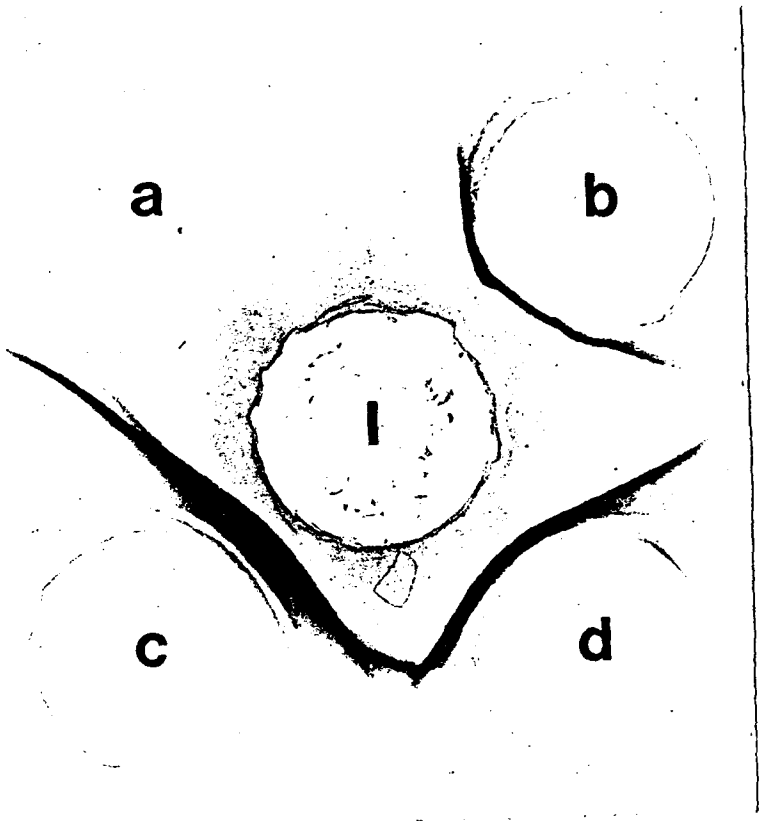
[b]One rat each point per experiment.

[c]Not determined.

FIGURE LEGENDS

FIG. 1. Immunodiffusion analysis of Green A column fractions using specific antiserum, a-DTW fraction; b-EL fraction; c-whole toxin B. anthracis; d-Michigan antigen; I-anti-PA antiserum; and II-anti-LF antiserum.

FIG. 2. SDS polyacrylamide gel electrophoresis of Green A column fractions. Lane 1-high molecular weight standards with Phosphorylase B MW 97,412 denoted by arrow; Lane 2-whole toxin B. anthracis; Lane 3-Michigan antigen; Lane 4-DTW; and Lane 5-EL. Acrylamide gel consisted of 10% acrylamide and 1% methylenebisacrylamide.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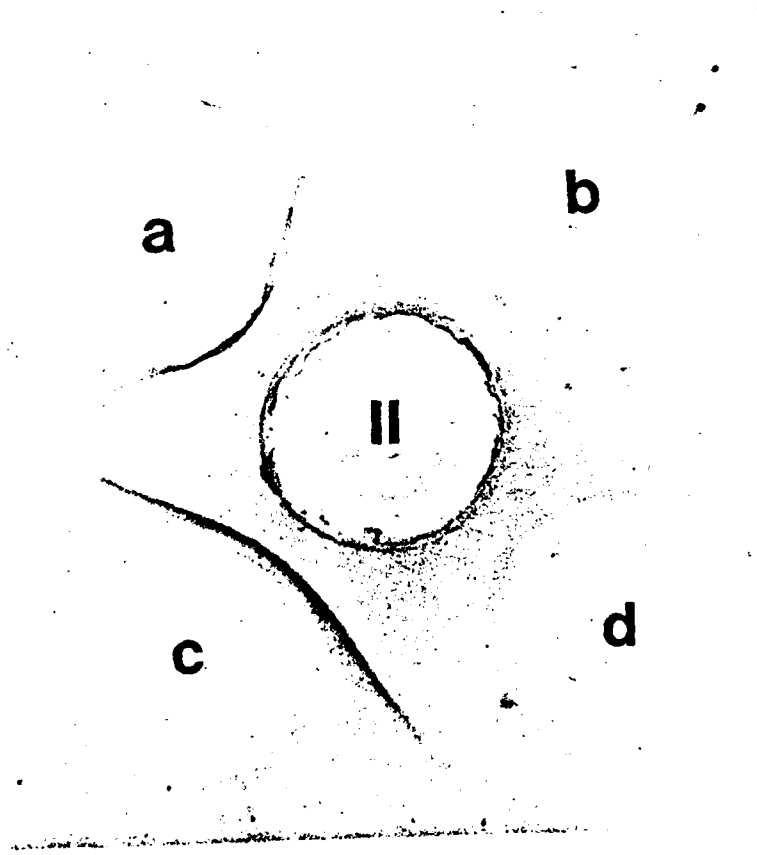
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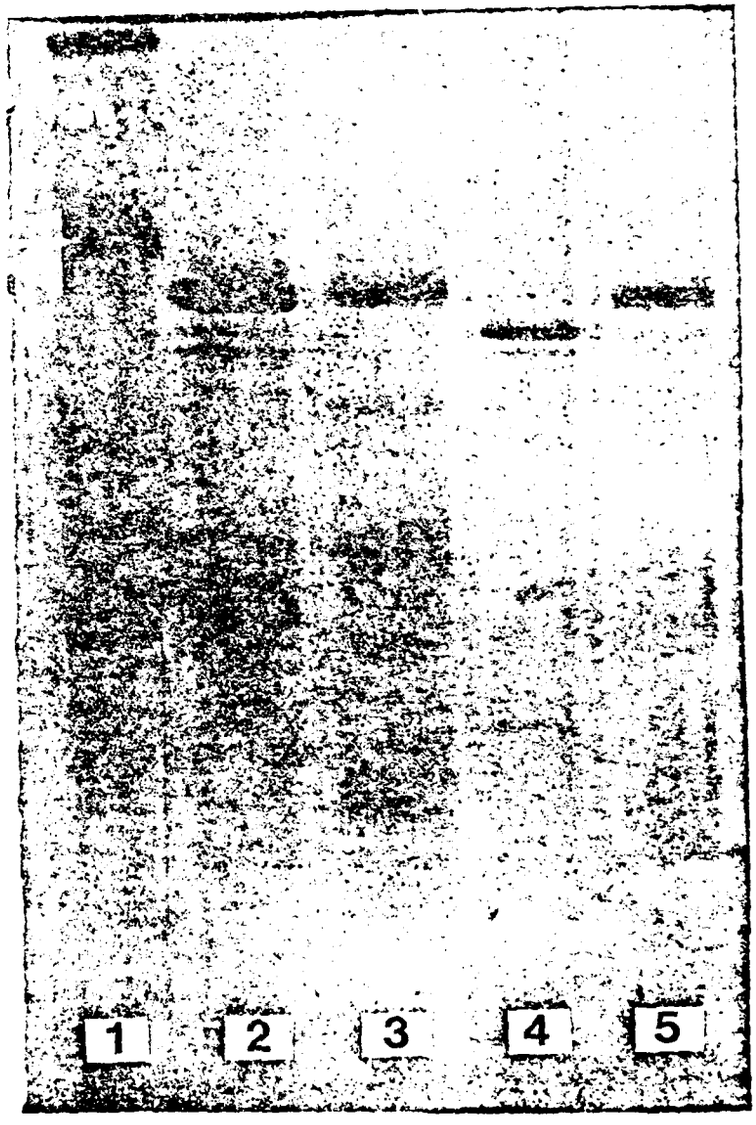
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